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Gamijeonssibaekchulsan regulates mast cell-mediated anaphylactic reaction

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Gamijeonssibaekchulsan (GJBS) is a typical Oriental medicine prescription which has been used in Korea for the treatment of allergic diseases and the development of physical strength. However, as yet there is no clear explanation of how GJBS affects the anaphylactic reaction and the immune function. In the present study murine models and MOLT-4 cells, a T cell line, were used to investigate these effects. Compound 48/80-induced systemic anaphylactic shock and ear swelling response were firstly analyzed. We also assayed histamine release and passive cutaneous anaphylaxis (PCA) in mice and cytokine productions in MOLT-4 cells. GJBS significantly inhibits compound 48/80-induced systemic anaphylactic shock and ear swelling response. GJBS also inhibits histamine release from rat peritoneal mast cells induced by compound 48/80. PCA activated by anti-dinitrophenyl immunoglobulin E is attenuated by GJBS. However, GJBS dose not affect the production of interferon-γ, interleukin (IL)-2, and IL-4 in MOLT-4 cells. These results indicate that GJBS has a potential regulatory effect on allergic reactions that are mediated by mast cells.

Keywords: gamijeonssibaekchulsan, histamine, passive cutaneous anaphylaxis, MOLT-4 cells

INTRODUCTION

Gamijeonssibaekchulsan (GJBS) is a typical Oriental medicine prescription which has been used in Korea for the treatment of allergic disease and the development of physical strength. To investigate how GJBS affects the anaphylactic reaction and immune function, we used murine models and T cell line (MOLT-4 cells).

In general, immediate-type hypersensitivity reactions that include urticaria, allergic rhinitis and asthma are mediated by various chemical mediators released from mast cells (Miescher & Vogel, 2002). Histamine, one of the substances released on degranulation of mast cells, is a well characterized and potent vasoactive mediator implicated in the acute phase of immediate-type hypersensitivity reactions (Moon *et al.,* 2004; 2005). Compound 48/80 is a well-known histamine releaser (Na *et al.,* 2004). Compound 48/80 also induces ear swelling in a skin anaphylactic reaction model. Ear swelling is a predictive response of dermal sensitization (Kim & Yang, 1999).

The secretory responses of mast cells can be induced by aggregation of their cell surface-specific receptors for immunoglobulin (Ig) E by the specific antigen (Metzger *et al.,* 1986; Alber *et al.,* 1991). The release of vasoactive substances is induced locally in naïve animals using IgE. IgE is injected intradermally into naïve animals and the tested substance

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Abbreviations: DNP, dinitrophenyl; FBS, fetal bovine serum; GJBS, gamijeonssibaekchulsan; HSA, human serum albumin; IL, interleukin; OPA, *o*-phthaldialdehyde; PCA, passive cutaneous anaphylaxis; RPMCs, rat peritoneal mast cells.

mixed with a dye (typically Evans blue) is subsequently administered intravenously (usually after 24–72 h). Subsequent to the antigen–antibody reaction, vasoactive substances are released, resulting in a dramatic local increase in vascular permeability (Bazin *et al.,* 1990). As the tested substance is mixed with a dye, the local cutaneous reaction is evidenced by a colored spot (Verdier *et al.,* 1994). The skin of mouse is a useful site for studying passive cutaneous anaphylaxis (PCA) (Jeong *et al.,* 2006).

Immunoregulatory cytokines play an important role in determining the nature and intensity of an immune response (Paul & Seder, 1994; Abbas *et al.,* 1996). Cytokines have been classified as Th1 types such as interleukin (IL)-2 and interferon (IFN)-γ or Th2 type IL-4, IL-5, IL-6 based on studies originally involving cloned murine $CD4^+$ T cell subsets (Mosmann *et al.,* 1986; Mosmann & Coffman, 1989). Th1 type cytokines promote cell-mediated immunity. Th2 cytokines primarily facilitate the development of humoral immune responses (Carter & Dutton, 1996; Stephens *et al.,* 2002). There are three classes of IFNs: interferon-α produced by leukocytes, interferon-β produced by fibroblasts, and interferon-γ produced by T cells. IFN-γ induces both humoral and cellular immunological response.

We used murine models to investigate how GJBS affects the systemic anaphylactic reaction, ear swelling response, histamine release, and passive cutaneous anaphylaxis (PCA). Moreover, we investigated the effects of GJBS on cytokine production in MOLT-4 cells.

MATERIALS AND METHODS

Materials. We purchased compound 48/80, anti-dinitrophenyl (DNP) IgE, DNP-human serum albumin (HSA), metrizamide, *o*-phthaldialdehyde (OPA), Evans blue, avidin-peroxidase, and 2,2-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) from Sigma Chemical Co. (St. Louis, MO, USA). In addition, we purchased α-minimal essential medium from Flow Laboratories (Irvine, UK); RPMI 1640, ampicillin, streptomycin, and fetal bovine serum (FBS) from Gibco BRL (Grand Island, NY, USA); anti-human IL-2, IL-4, and IFN-γ antibodies, biotinylated anti-human IL-2, IL-4, and IFN-γ antibodies, and recombinant (r) human IL-2, IL-4, and IFN-γ from R&D Systems (Minneapolis, MN, USA).

Animals. We purchased original stock of male ICR mice (4 weeks old) and male Wistar rats (7 weeks old) from Dae-Han Experimental Animal Center (Eumsung, Chungbuk, Republic of Korea) and maintained at the College of Pharmacy, Wonkwang University. The animals were housed three to five per cage in a laminar air-flow room maintained at a temperature of 22±1°C and relative humidity of 55±10% throughout the study. No animal was used more than once. The research was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in US guidelines (NIH publication #85-23, revised in 1985).

Preparation of GJBS. GJBS extract which is a mixture of twenty five traditional drugs obtained from the Oriental Pharmacy, College of Oriental Pharmacy (Iksan, Republic of Korea). The ingredients of 120 g GJBS include 10 g of Astragali Radix (*Astragalus membranaceus* Bunge), 10 g of Taraxaci Herba (*Taraxacum platycarpum* H. Dahlstedt), 10 g of Ulmi Cortex (*Ulmus macrocarpa* Hance), 6 g of Atractylodis Rhizoma Alba (*Atractylodes japonica* Koidzumi), 6 g of Glycyrrhizae Radix (*Glycyrrhiza uralensis* Fischer), 6 g of Coicis Semen (*Coix lacryma-jobi* Linné var. *ma-yuen* Stapf), 6 g of Dioscoreae Rhizoma (*Dioscorea batatas* Decaisne), 5 g of Puerariae Radix (*Pueraria lobata* Ohwi), 4 g of Hoelen (*Poria cocos* Wolf), 4 g of Agastachis Herba (*Agastache rugosa* O. Kuntze), 4 g of Tricosanthis Radix (*Trichosanthes kirilowi* Maximowicz), 4 g of Magnoliae Flos (*Magnolia denudata* Desrousseaux), 4 g of Xanthii Fructus (*Xanthium strumarium* Linné), 4 g of Paeoniae Radix (*Paeonia lactiflora* Pallas), 4 g of Myristicae Semen (*Myristica fragrans* Houttuyn), 4 g of Mume Fructus (*Prunus mume* Siebold et Zuccarini), 4 g of Platycodi Radix (*Platycodon grandiflorum* A. De Candolle), 4 g of Citri Unshii Pericarpium (*Citrus unshiu* Markovich), 3 g of Ginseng Radix Alba (*Panax ginseng* C. A. Meyer), 3 g of Aucklandiae Radix (*Aucklandia lappa* Decne.), 3 g of Zanthoxyli Pericarpium (*Zanthoxylum piperitum* De Candolle), 3 g of Angelicae Gigantis Radix (*Angelica gigas* Nakai), 3 g of Ponciri Fructus (*Poncirus trifoliata* Rafinesqul), 3 g of Cimicifugae Rhizoma (*Cimicifuga heracleifolia* Komarov), 3 g of Bupleuri Radix (*Bupleurum falcatum* Linné). Extract of GJBS was prepared by decocting the dried prescription of herbs with boiling distilled water (GJBS/water=1:5, w/v). The extraction decocted for approx. 3 h was filtered, lyophilized, and kept a 4°C. Dilutions were made in saline then filtered through 0.22-μm syringe filter.

Compound 48/80-induced systemic anaphylactic reaction. Mice (n=5) were given an intraperitoneal injection of the mast cell degranulator compound 48/80 (8 mg/kg). GJBS was dissolved in saline and administered orally with a sonde 1 h before the injection of compound 48/80. Mortality was monitored for 22 min after induction of anaphylactic reaction.

Ear swelling response. Compound 48/80 was freshly dissolved in saline and injected intradermally (100 μg/site) into the dorsal side of a mouse (n=5) ear using a microsyringe with a 28-gauge hypodermic needle. Ear thickness was measured with a

digimatic micrometer (Mitutoyo, Japan) under mild anesthesia. Ear swelling response corresponded to an increase of thickness above baseline control values. Ear swelling response was determined 40 min after compound 48/80 or vehicle injection. GJBS (0.01 to 1 g/kg) was administered orally 1 h before the compound 48/80-injection. The values obtained appear to represent the effect of compound 48/80 rather than the effect of vehicle injection (physical swelling), since the ear swelling response evoked by physiologic saline returned to almost baseline thickness within 40 min.

Preparation of rat peritoneal mast cells (RPMCs). RPMCs were isolated as previously described (Jippo-Kanemoto *et al.,* 1993). In brief, rats were anesthetized with ether, and injected with 20 ml of Tyrode buffer B (134 mM NaCl, 5 mM glucose, 12 mM $NaHCO_3$, 2.9 mM KCl, 0.34 mM NaH_2PO_4) containing 0.1% gelatin (Sigma) into the peritoneal cavity; the abdomen was gently massaged for about 90 s. The peritoneal cavity was carefully opened, and the fluid containing peritoneal cells was aspirated with a Pasteur pipette. Then the peritoneal cells were sedimented at $150 \times g$ for 10 min at room temperature and resuspended in Tyrode buffer B. Mast cells were separated from the major components of rat peritoneal cells (i.e., macrophages and small lymphocytes) according to the method described by Yurt *et al.* (1977). In brief, peritoneal cells suspended in 1 ml of Tyrode buffer B were layered onto 2 ml of 0.225 g/ml metrizamide (density 1.120 g/ml; Sigma) and centrifuged at room temperature for 15 min at $400 \times g$. The cells remaining at the buffer-metrizamide interface were aspirated and discarded; the cells in the pellet were washed and resuspended in 1 ml of Tyrode buffer A containing calcium (10 mM Hepes, 130 mM NaCl, 5 mM KCl, 1.4 mM $CaCl_2$, 1 mM $MgCl_2$, 5.6 mM glucose, 0.1% bovine serum albumin). Mast cell preparations were about 95% pure as assessed by toluidine blue staining. More than 97% of the cells were viable as judged by the Trypan blue uptake.

Histamine assay. Purified RPMCs were resuspended in Tyrode buffer A containing calcium for the treatment with compound 48/80. RPMC suspensions (2×10^5 cells/ml) were preincubated for 10 min at 37°C before the addition of compound 48/80 for stabilization. The cells were preincubated with GJBS (0.01 to 1 mg/ml) for 30 min, and then incubated for 20 min with compound 48/80 (6 μg/ml). The reaction was stopped by cooling the tubes in ice. The cells were separated from the released histamine by centrifugation at $400 \times g$ for 5 min at 4°C. Residual histamine in the cells was released by disrupting the cells with perchloric acid and centrifugation at $400 \times g$ for 5 min at 4°C. The histamine content was measured by the OPA spectrofluorometric procedure of Shore *et al.* (1959). The fluorescent intensity was measured at 440 nm (excitation at 360 nm) in a spectrofluorometer. The inhibition percentage of histamine release was calculated using the following equation:

$$\% \text{ inhibition} = (A - B) \times 100/A$$

where A is histamine release without GJBS and B is the histamine release with GJBS.

PCA. IgE-dependent cutaneous reaction was generated by sensitizing the skin with an intradermal injection of anti-DNP IgE followed 48 h later with an injection of DNP-HSA into the mice tail vein. The DNP-HSA was diluted in phosphate-buffered saline (PBS). The mice were injected intradermally with 100 ng of anti-DNP IgE into each of three dorsal skin sites that had been shaved 48 h earlier. The sites were outlined with a water-insoluble red marker. Forty-eight hours later, each mouse received an injection of 200 μl of a 1 : 1 mixture of 1 mg/ml DNP-HSA in PBS and 4% Evans blue *via* the tail vein. One hour before this injection, GJBS was administered orally with a sonde. The mice were sacrificed 40 min after the intravenous challenge. The dorsal skin of the mouse was removed for measurement of the pigment area. The amount of dye was then determined colorimetrically after extraction with 0.5 ml of 1 M KOH and 4.5 ml of a mixture of acetone and phosphoric acid (in a ratio of 13:5, v/v), based on the method of Katayama *et al.* (1978). The absorbance of the extract was measured at 620 nm in a spectrophotometer, and the amount of dye was calculated using an Evans blue calibration curve.

MOLT-4 cell culture. T cell line MOLT-4 cells were grown in RPMI 1640 medium (Gibco BRL, USA) supplemented with 10% FBS (JRH Bioscience, USA), 100 U/ml penicillin and 100 μg/ml streptomycin at 37°C in the presence of 5% CO_2.

Cytokines assay. The MOLT-4 cells were treated with various concentrations of GJBS (0.01 to 1 mg/ml) for 24 h. We then used the enzyme-linked immunosorbent assay (ELISA) method to assay the culture supernatants for the IFN-γ, IL-2, and IL-4 protein levels. To measure the cytokines, we used a modified ELISA method (Moon *et al.,* 2007a). To measure the color development at 405 nm, we used an automated microplate ELISA reader.

Statistical analysis. Our results are expressed as the mean ± standard error of the mean (S.E.M.). The statistical evaluation of the results was performed by an independent *t*-test and an analysis of variance with a Tukey *post hoc* test. The results are significant with a value of $P < 0.05$.

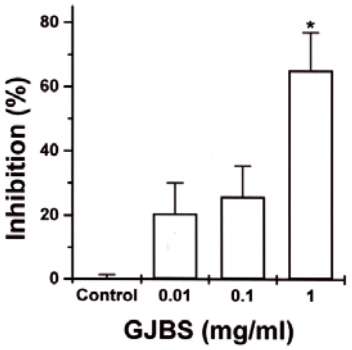

Figure 1. Effect of GJBS on compound 48/80-induced histamine release from RPMCs.
RPMCs (2×10^5 cells) were preincubated with various concentrations of GJBS at 37°C for 10 min prior to incubation with compound 48/80. Results represent the mean ± S.E.M. of three independent experiments. $^*P<0.05$; significantly different from the control value.

Table 1. Effect of GJBS on compound 48/80-induced systemic anaphylactic reaction in mice.

Treatment	Dose (g/kg)[a]	Compound 48/80 (8 mg/kg)[b]	Mortality (%)[c]
None (saline)	–	+	100.0±0.0
GJBS	0.01	+	40.0±10.0
	0.1	+	20.0±5.0*
	1	+	20.0±5.0*
	1	–	0.0±0.0

[a]Groups (n = 5/group) of mice were orally pretreated with 200 μl of saline or GJBS. GJBS was given at various doses 1 h before compound 48/80 injection. [b]Compound 48/80 solution was given intraperitoneally to groups of mice. [c]Mortality (%) is presented as the 'number of dead mice ×100 / Total number of experimental mice'. Each value is the mean of three independent experiments. *$P<0.05$; significantly different from the control value.

RESULTS

Effect of GJBS on compound 48/80-induced systemic anaphylaxis

To assess the contribution of GJBS in anaphylactic reactions, we first used an *in vivo* model of systemic anaphylaxis. As a nonimmunologic stimulator, compound 48/80 (8 mg/kg) was used. After the injection of compound 48/80, the mice were monitored for 22 min, after which the mortality rate was determined. The period for observation of mortality was based on the control mice that had died in 22 min by compound 48/80. As shown in Table 1, an oral administration of saline as a control resulted in a fatal reaction in 100% of each group. When GJBS was orally administered at the doses of 0.1 and 1 g/kg 1 h before compound 48/80 injection, the mortality was significantly inhibited ($P<0.05$; Table 1).

Effect of GJBS on ear swelling response

The fact that intradermal application of compound 48/80 at 50 to 200 μg/site can induce an ear swelling response in normal mice was confirmed in a previous experiment (Moon *et al.*, 2007b). We chose a concentration of 100 μg/site for compound 48/80-induced optimal ear swelling response in this experiment. As shown in Table 2, when mice were pretreated with GJBS for 1 h, the ear swelling responses to compound 48/80 were significantly inhibited (1 g/kg GJBS; $P<0.05$).

Effect of GJBS on histamine release from RPMCs

The inhibitory effect of GJBS on compound 48/80-induced histamine release from RPMCs is shown in Fig. 1. GJBS significantly inhibited compound 48/80-induced histamine release at the dose of 1 mg/ml ($P<0.05$). The histamine fluorescence intensity at the doses of 0.01, 0.1, and 1 mg/ml was 26.82±3.34, 24.92±3.38, and 13.00±3.71, respectively. Control and spontaneous values were 33.14±0.78 and 1.69±0.59.

Effect of GJBS on PCA

PCA is one of the most important *in vivo* models of anaphylaxis in local allergic reactions

Table 2. Effect of GJBS extract on compound 48/80-induced ear swelling response in mice.

The mice were orally administered with the indicated concentration of GJBS 1 h prior to the compound 48/80 application. Twenty microliters of compound 48/80 (100 μg/site) were injected intradermally. Results represent the mean ±S.E.M. of three independent experiments. *$P<0.05$; significantly different from the control value.

Treatment	Dose (g/kg)	Pre-thickness (mm)	Post-thickness (mm)	Inhibition (%)
Control	–	0.34±0.03	0.74±0.01	0.00±7.09
GJBS	0.01	0.30±0.00	0.67±0.02	9.45±5.90
	0.1	0.31±0.02	0.63±0.01	19.33±2.31
	1	0.32±0.02	0.62±0.03	26.83±2.14*

(Wershil *et al.*, 1987). Local injection of anti-DNP IgE followed by an intravenous antigenic challenge was performed. Anti-DNP IgE was injected into dorsal skin sites. After 48 h, all animals were injected intravenously with DNP-HSA containing Evans blue dye. The cutaneous anaphylactic reaction was best visualized by the extravasation of the dye. When GJBS was orally administered to the mouse at the doses of 0.01 to 1 g/kg, the PCA was significantly and dose-dependently inhibited ($P<0.05$; Fig. 2). The maximal inhibition rate was 46.41±3.50 at 1 g/kg GJBS.

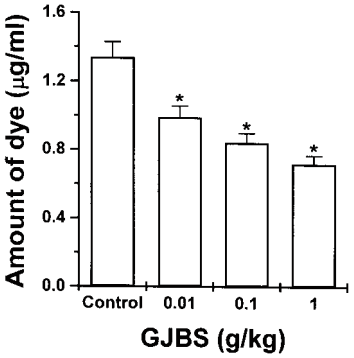

Figure 2. Effect of GJBS on 48 h PCA in mice.
GJBS was administered orally 1 h prior to the challenge with antigen (DNP-HSA). Results represent the mean ± S.E.M. of three independent experiments (n=5/group). $^*P<0.05$; significantly different from the control value.

Effect of GJBS on the productions of IFN-γ, IL-2, and IL-4 in MOLT-4 cells

To assess the effect of GJBS on the production of cytokines, MOLT-4 cells were treated with various concentrations of GJBS for 24 h. The levels of IFN-γ, IL-2, and IL-4 were analyzed by ELISA. As shown in Fig. 3, the effect of GJBS on the production of cytokines in MOLT-4 cells was not significant.

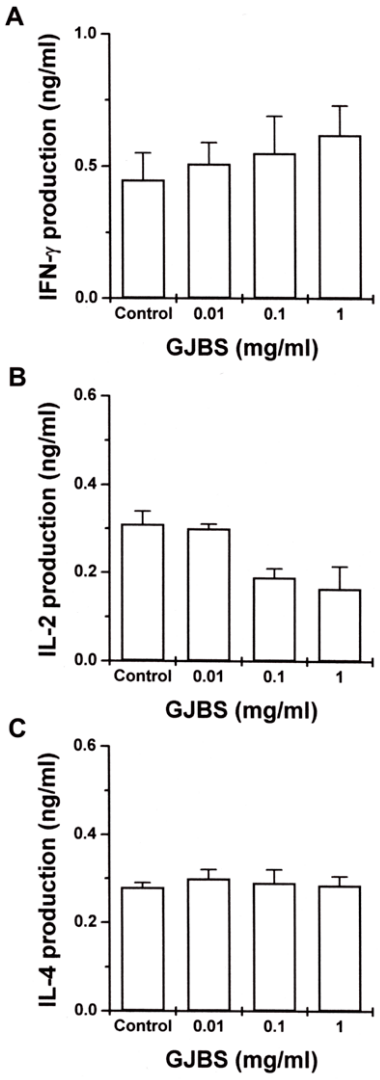

Figure 3. Effect of GJBS on IFN-γ, IL-2, and IL-4 production in MOLT-4 cells.
Culture supernatants were collected from saline- or GJBS-treated MOLT-4 cells cultured for 24 h. Cytokine levels in culture supernatants were measured using ELISA. A. IFN-γ production; B. IL-2 production; C. IL-4 production.

DISCUSSION

Stimulation of mast cells with compound 48/80 is believed to initiate the activation of a signal transduction pathway which leads to histamine release. There have been some reports that compound 48/80 is able to activate G proteins (Mousli *et al.*, 1990a; 1990b). Chadi *et al.* (2000) announced that compound 48/80 activates mast cell phospholipase D (PLD) *via* heterotrimeric GTP-binding proteins. They observed that recombinant $G_{\beta 2\gamma 2}$ subunit markedly synergized PLD activation by compound 48/80 in permeabilized RBL-2H3 cells. Murine mast cells are a good experimental model for the study on compound 48/80-induced histamine release (Alfonso *et al.*, 2000). Tasaka *et al.* (1986) reported that compound 48/80 increased the permeability of the lipid bilayer membrane by causing a perturbation of the membrane. That report indicates that the membrane permeability increase may be an essential trigger for the release of mediators from mast cells. We have shown that GJBS inhibits systemic anaphylactic reaction, ear swelling response, and histamine release (Tables 1 and 2, Fig. 1). Thus, it is possible to hypothesize that GJBS might act on the lipid bilayer membrane preventing the perturbation induced by compound 48/80.

The high-affinity IgE-receptor (FcεRI) is expressed primarily by mast cells (Daeron, 1997). Cross-linking of IgE-bound FcεRIs by a multivalent Ag on mast cells induces the release of biologically active mediators: the preformed mediators stored in the cytoplasmic granules, including histamine and β-hexosaminidase, and the newly synthesized me-

diators, such as leukotrienes and cytokines (Galli *et al.*, 2005a; 2005b). To reproduce these reactions in animals, a PCA model was used in passively sensitized mice. Our results showed that GJBS dose-dependently inhibits the PCA (Fig. 2). It is conceivable that GJBS inhibits the immediate type allergic reactions, probably through interference with the degranulation system.

Immunoregulatory cytokines play an important role in determining the nature and strength of an immune response (Paul & Seder, 1994; Abbas *et al.*, 1996). Recent studies indicate that the ratio of the two cytokine types, Th1 and Th2, is closely correlated with the outcome of many diseases, and controlling the Th1/Th2 ratio has been demonstrated as a therapeutic strategy for various diseases (Singh *et al.*, 1999; Boothby *et al.*, 2001; Spellberg & Edwards, 2001). Many cancer vaccines, particularly in combination with immune adjuvants, elicit strong cellular immune responses, leading to the production of Th1-type cytokines, such as IFN-γ, IL-2, and TNF-α (Dalgleish, 2000). IFN-γ is also an important cytokine in the host defense against infection by viral and microbial pathogens (Samuel, 2001). Previously, we reported that Th2 cytokine levels were higher than Th1 cytokine levels in various diseases, including cerebral infarction, allergies, and asthma (Kim *et al.*, 2000; Jeong *et al.*, 2002). Contrary to our expectation in this study, GJBS does not affect the production of IFN-γ, IL-2, and IL-4 in MOLT-4 cells (Fig. 3). We can therefore deduce that GJBS dose not affect the T cell-induced cytokines production but regulates the anaphylactic reactions by a direct effect on mast cells.

In conclusion, we have shown that GJBS can regulate the anaphylactic reactions that are mediated by mast cells. Overall, our results suggest that GJBS may have a clinical applicability to the allergic disorders.

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